Tracing thymic output in older individuals

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Summary

As a result of age-associated thymic atrophy, T cell production declines with age. Some studies suggest that production undergoes an exponential decline starting at birth, while others consider the decline to be in a biphasic manner with a rapid reduction in output occurring before middle age followed by a phase in which output declines at a regular, albeit much slower, rate. Both approaches provide estimations of the time of termination of thymic output, but on the basis of limited amounts of data. We have analysed blood from more than 200 individuals between the ages of 58 and 104 years to determine changes in thymic output using signal-joint T cell receptor excision circles (sjTREC)/T cells as our measure. To reduce any potential geographical or nutritional bias we have obtained samples from five different European countries. Our results reveal that while the absolute number of T cells per microlitre of blood does not change significantly across the age range we tested, the values of sjTREC per microlitre show wide variation and reveal an age-associated decline in thymic output. In addition we show gender differences, with notably higher thymic output in females than males at each decade. More importantly, we noted a significant decline in sjTREC/T cell levels in those more than 90 years of age in both males and females. Our results provide information about the potential end-point for thymic output and suggest that sjTREC analysis may be a biomarker of effective ageing.

Keywords: elderly, mortality, thymic output, thymus TREC, TREC assay

Introduction

Epidemiological surveys, clinical observations and laboratory tests all reveal that the immune system declines with age. Indications of this decline include a poorer response to vaccination [1], a higher prevalence of certain cancers associated with viral infections [2], an increased susceptibility to infections [3] and a higher likelihood of being infected by emerging pathogens than younger individuals [4]. In addition, older individuals often show an increased difficulty in dealing with pathogens which they have overcome previously. Common problems include reactivation of persistent viruses such as herpes zoster [5] or cytomegalovirus [6] and also a disproportionate immune response to the latter [7]. The elderly also experience more problems than younger individuals following the yearly return of influenza and respiratory syncytial virus (RSV) [8]. Infection with influenza in younger individuals is followed normally by a disease limited in its duration to 1–2 weeks, but the consequences of infection in the elderly differ, being more likely to progress to chronic illness and an irreversible loss of physical condition [9].

Because of their central role in orchestrating the immune response, the age-associated changes noted in immune function have been linked to altered functional characteristics of individual T cells and also to modifications occurring in the peripheral T cell pool [10]. It has not been proved formally that changes in T cell function observed with advancing age are completely disconnected from the consequences of modification in the peripheral T cell pool, as events such as proliferation induced by the homeostatic milieu of the ageing organism may contribute to the reduced functional capacity of T cells [11].

A potential driver of age-related changes in the peripheral T cell pool is atrophy of the thymus. A reduction in thymic activity is a feature of ageing in mammals. In humans, fat accumulates in the thymus throughout life [12] reducing the active areas of thymopoiesis, and this contributes to a decline in the output of T cells. Measurement of this decline in previous studies has produced different views on the kinetics

of this process. Some studies indicate an exponential decline [13] with T cell output beginning early in life and estimated to terminate at approximately 75 years of age [14]. Others suggest that the thymus atrophies in a biphasic manner [15] with the initial phase beginning early in life, at least as early as the first year and proceeding at a rate of 3% per year until middle age. Thereafter the rate changes to a constant rate of 1% per year, leading to the estimated total loss of thymic tissue by 105 years of age [16,17].

Recent work shows that the reversal of thymic atrophy is a viable option, but the timing of when such a procedure should begin would be critically dependent upon determining the period when thymic output ceases. In order to provide more information about the decrease in thymic output later in life we analysed samples collected from 215 healthy elderly individuals, with ages ranging from 60 to 100 years, and to reduce any bias related to environmental factors and/or lifestyle we obtained samples from participating centres across five European countries (France, Germany, Greece, Italy and Poland)) [18]. We quantified changes in thymic output using signal-joint T cell receptor excision circles (sjTRECs) per T cells measured by real-time polymerase chain reaction (PCR), as described previously [19].

Materials and methods

Sample collection and storage and analysis

Peripheral blood (PB) samples were collected from healthy elderly individuals from participating centres across five European countries (France, Germany, Greece, Italy and Poland) [18]. Informed consent was obtained from healthy adult volunteers, with ages ranging from 58–104 years. Peripheral blood mononuclear cells (PBMC) were isolated and the samples were stored at −140°C until required for analysis. Frozen PBMC were thawed and an aliquot containing 1×10^5 cells stained with phycoerythrin (PE)-conjugated anti-CD3 (BD Bioscience, Oxford, UK) according to the manufacturer's instructions. The T cell measurement was determined using a fluorescence activated cell sorter (FACS-)Calibur (Becton Dickinson) flow cytometer. DNA was extracted from the remaining cells using the Puregene DNA purification kit (Flowgen, Ashby de la Zouch, UK). The DNA was stored at −20°C until required for analysis. When the DNA was thawed its concentration was determined by optical density readings using a spectrophotometer and aliquots of 50 ng was removed for use in real-time PCR experiments.

Real-time PCR assessment of sjTREC and albumin

Human sjTREC and albumin (ALB) levels were quantified using real-time PCR performed on the Roche Light Cycler (Roche Diagnostics, Lewes, UK). A PCR reaction mixture containing 50 ng of DNA, 0·5 μM of forward and reverse primers and 2× SYBR Green mix (Qiagen, Crawley, UK) in a

final reaction volume of 10 μl, using sterile water. The primer sequences used were sjTREC forward: GGC AGA AAG AGG GCA GCC CTC TCC AAG and reverse: GCC AGC TGC AGG GTT TAG G or ALB forward: CTA TCC GTG GTC CTG AAC CAG TTA TG and reverse: CTC TCC TTC TCA GAA AGT GTG CAT AT, which produced amplicons of 195 base pairs (bp) and 206 bp, respectively. Real-time PCR conditions on the Light Cycler were 95°C for 15 min, followed by 45 cycles at 95°C for 15 s, 61°C for 30 s and 72°C for 20 s (fluorescent acquisition). The albumin reaction was performed as described above, except that the annealing temperature was changed to 60°C. The 195 bp and 206 bp PCR products were identified by melting-point analysis. A standard curve generated from a serial dilution of known concentration of sjTREC or albumin plasmid was used to enable calculation of the number of detectable molecules from the test samples. The copy number of sjTREC and ALB (x) was calculated using the following equations: $y_{sjTREC} = -3·468x + 42·09$ and $y_{ALB} = -3·374x + 40·593$, where the cycle threshold (C_t) value is substituted as y. A standard concentration of 1×10^4 sjTREC or ALB molecules was included to determine variance between each run and comparability of the sample. All samples were run in duplicate and an average of the result used for statistical analysis. Where C_t values of the duplicates were greater than 1·5 cycles the samples were rerun.

From these readings we obtained a value of sjTREC per 50 ng of DNA. The amount of DNA obtained from the sample of PBMC was known, so we could calculate the number of sjTREC in the PBMC sample. Because sjTREC can be derived only from T cells and we had determined the number of CD3+ T cells by immunophenotyping in the sample, we could ascribe a definite value of sjTREC/T cell to the sample.

Statistical analysis

The results of the descriptive analysis are presented for numerical variables in the form of means ± standard deviation (s.d.) and median for age; sample sizes and percentages calculated for categorical outcomes. Subjects' characteristics and blood sample components were compared with respect to the age group. Statistical tests used for the comparative analysis were chosen according to the type of variable, the sample size under consideration and the number of group compared. Thus, numerical outcomes were tested using the Kruskal–Wallis test (when more than two groups) and the Mann–Whitney test (when two groups); Fisher's exact test was used for categorical outcomes. The level of significance was set at $P = -0·05$. In addition, linear Pearson correlation coefficients (r) were calculated using a linear regression model to measure the strengths and directions of the linear relationships between the number of TREC and the different age groups. These statistical analyses were performed using StatView (version 5·0; SAS Institute, Inc., Cary, NC, USA). Graph were drawn using Microsoft Office Excel© or

GraphPad Prism (version 4·0 for Windows; GraphPad Software, San Diego, CA, USA; http://www.graphpad.com).

Results

Refining sample analysis according to DNA integrity

We were aware that any indication of relative changes in sjTREC values in the samples could be compromised through a loss of integrity of the DNA. In order to ensure equivalence we analysed in excess of 250 samples and selected those for further analysis on the basis of their DNA integrity as determined by the amplifiability of the albumin gene [20,21]. Any sample with a C_t value greater than 24·0 cycles, which approximates to fewer than 1×10^5 albumin molecules, was excluded from further analysis. Of the samples analysed approximately 17% were deemed unacceptable after albumin amplification, therefore we were able to identify 215 samples for further analysis. Surprisingly, a higher than expected proportion of unacceptable samples fell within the 80–89 age group, which is reflected as an apparent gap between 85 and 89 years.

sjTREC values, leucocytes and gender

Analysis of the sjTREC per 10^5 T cells in our population (Fig. 1) showed a slow decline in their numbers between the 6th and 9th decade of life, with the most pronounced decline seen in those individuals more than 90 years of age. Interdecade comparison of the sjTREC levels revealed that individuals in their 10th decade had significantly lower levels ($P < 0.05$) than those obtained from individuals in the 7th, 8th and 9th decades (P-values of 0·0002, 0·0004 and < 0.0001, respectively). Moreover, samples from these earlier decades showed a wide range of values (see Table 1).

Because of concerns that these results were due to changes either in the number of leucocytes or the number of $CD3^+$ T cells in the blood of our donors [22] we analysed both of these parameters. Comparative analysis revealed no significant change across the age range (see Table 1), either in the number of leucocytes ($P > 0.05$) or in the absolute number of T cells ($P > 0.05$) as depicted in Fig. 2.

Previous work has shown differences in sjTREC levels due to gender [23] The sex ratio measured in the present sample was near to 1, with approximately 52% (113 of 215) being females. In Fig. 3 the overall decline seen in both males and females highlights that females had higher levels of detectable sjTREC per 10^5 T cells compared to males at all age groups. However, this difference was not significant across the entire age range, but only between the males and females sampled in the 70–79 age range ($P < 0.03$).

sjTREC distribution during separate decades

It is common to report T cell values as the number of T cells/ml and use this value to set defined ranges and differ-
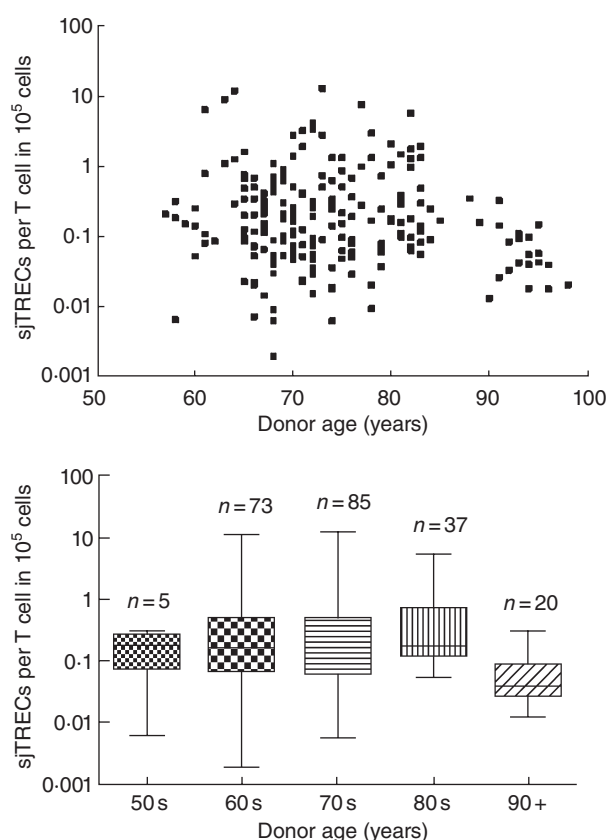

Fig. 1. Age-related changes in T cell receptor excision circles (TRECs) per 10^5 T cells. Each dot represents an individual measurement.

ences between ages [24]. In order to normalize the values with respect to T cells numbers in the blood we calculated the values of the number of $sjTREC^+$ cells per ml of blood and then compared this with the values we obtained for leucocyte numbers and T cell numbers in the 7th–10th decades (Table 1). The results confirm the maintenance of both leucocyte and T cell numbers in the blood over the entire age range and also provide further confirmation of the degree of variation in the numbers of $sjTREC^+$ cells. Interestingly, there is an observable convergence of the overall spread of the sjTREC levels with advancing age in the decades analysed. The standard deviations of the values progress from almost three times the average value in the 7th decade to twice the value in the next decade to almost equal to the value in the 9th decade. During these decades the average sjTREC/ml remains fairly steady between the ages of 60 and 89 at about 0·0006% of the T cells.

The greatest change occurs in the 10th decade, and the degree of change is understood more easily if we calculate changes within the T cell pool as a whole. To do this we need to assume that each sjTREC is present as a single entity in a T cell (i.e. no doubles), that the blood volume in these individuals is close to average at 5 litres [25] and that approximately 2% of the total T cell pool resides within the blood [26]. From this we calculate that the average individual in

Table 1. Leucocytes, T cell and T cell receptor excision circle (TREC) values.

	7th decade (age 60–69) $n = 73$	8th decade (age 70–79) $n = 85$	9th decade (age 80–89) $n = 37$	10th decade (age > 90) $n = 20$
Age (years)				
Mean ± s.d.	66·18 ± 2·43	73·75 ± 2·80	82·22 ± 1·99	93·50 ± 2·01
Median	67	74	82	93·5
Gender				
Female % (*n*)	49·0% (36)	48·2% (41)	54·0% (20)	60·0% (12)
Leucocytes (no./μl)				
Mean ± s.d.	6 069 ± 1 572	6 319 ± 1 725	6 732 ± 1 527	6 338 ± 1 685
T cells (no./μl)				
Mean ± s.d.	1 320 ± 502	1 211 ± 471	1 383 ± 566	1 064 ± 407
TRECs (no./ml)				
Mean ± s.d.	8·34 ± 22·09*	7·73 ± 16·37*[§]	9·38 ± 12·63*	0·60 ± 0·44
Total TRECs in blood[†]	41 707	38 653	46 927	3 008
Total TRECs in body[+]	2 085 364	1 932 671	2 346 388	150 400

*Indicates a significant difference when compared to the 10th decade group ($P < 0·05$). [†]Extrapolation of total TRECs in the blood based on 5 litres circulation blood volume. [+]Extrapolation of TREC numbers in the body based on 2% of T cell pool in the circulating blood [26]. [§]Indicates a significant difference in TREC numbers observed between males and females within this age range ($P < 0·05$). s.d.: standard deviation; no.; number.

their 9th decade has $2·35 \times 10^6$ sjTRECs in their T cell pool and that this value drops to $1·5 \times 10^5$ in the 10th decade. This is a dilution factor of almost fivefold without a comparative change in the overall T cell numbers.

Using a linear regression model, further analysis was performed of the observed decline in sjTREC level as a function of age. Table 2 highlights the relationship between sjTREC levels and increasing age. Across the entire age range no significant correlation was observed; however, as the transition is made from the 8th to 9th decades a significant corre-

lation coefficient is seen of $r = −0·285$ ($P = 0·05$), progressing to $−0·463$ ($P = 0·02$) by the 10th decade.

Discussion

We have analysed thymic output through following the change in the sjTREC values in the peripheral blood CD3^{+} T cells of more than 200 individuals from five different European countries who were within the age range 60–100 years. Our results provide information about the potential endpoint for thymic output and also provide a suggestion that sjTREC analysis may prove to be a biomarker of ageing. The observed convergence of the sample heterogeneity in the sjTREC levels with increasing age raises a number of interesting possibilities. First, are low sjTREC measurements reflective of an individuals immunosenescence status; if so, are the individuals in the lower left quadrant of Fig. 4 (low
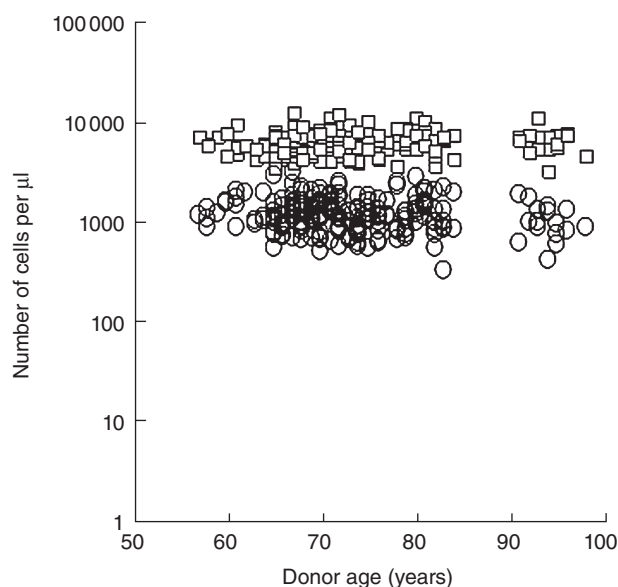

Fig. 2. Number of leucocytes (□) and CD3^{+} T cells (○) per microlitre of blood at different ages.
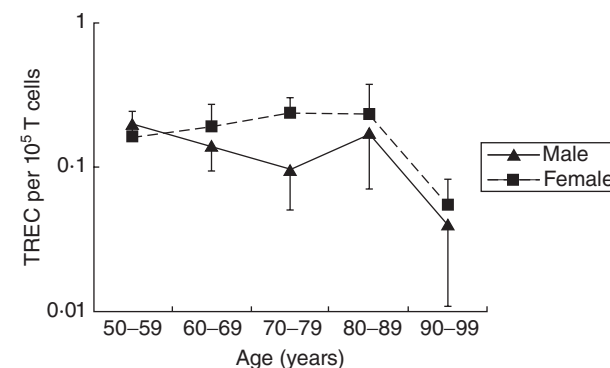

Fig. 3. Age-related changes in T cell receptor excision circles (TRECs) per 10^5 T cells according to gender.

Table 2. Pearson correlation coefficients (*r*) calculated by regression linear model describing the relationship between the number of T cell receptor excision circles (TRECs)/10^5 T cells and subjects' age

Age group (years)	Sample size (*n*)	Correlation coefficient (*r*)*	*P*-value†
7th to 10th decades	215	−0·142	n.s.
7th decade	73	−0·109	n.s.
8th decade	85	−0·0554	n.s.
9th decade	37	−0·442	0·03
10th decade	20	−0·497	0·02
7th and 8th decades	158	−0·0989	n.s.
7th, 8th and 9th decades	195	−0·0946	n.s.
8th and 9th decades	122	−0·285	0·05
8th, 9th and 10th decades	142	−0·382	0·04
9th and 10th decades	57	−0·463	0·02

r: Expresses the degree that, on an average, two variables change correspondingly. The *r*-value is a number between −1 and 1; *r*-value: 0 indicating there is a random, non-linear relationship between the two variables. Positive and negative signs indicate the direction of a linear relationship between two variables. †*P*-value: the probability that *r* being not different from zero. If it is lower than < 0·05 the correlation coefficient calculated is statistically significant. n.s.: not statistically significant (*P*-value > 0·05).

sjTREC level at younger age) at a more advanced stage of immunosenescence? The converse argument could also be inferred for individuals with the highest sjTREC levels (upper left quadrant). These individuals may therefore be more likely to progress to become the long-lived healthy individuals observed in the low right quadrant. This concept lends itself to the argument that immunosenescence is not merely a measurement of chronological age, but points towards immune exhaustion arising at different ages. The downward trajectory of an individual's thymic output profile over time has been demonstrated previously by Kilpatrick *et al.* [27] and could be considered as part of longitudinal studies similar to the Swedish OCTA and NONA studies [28,29] to investigate further the potential role of sjTREC as predictive markers of ageing.

Age-associated decline in immune function can be demonstrated clinically by changes in the prevalence of infectious disease within the elderly and can be evaluated in laboratory tests by the decreased functional capability of lymphocytes [30]. Some of this functional decline may be attributed to the accumulation of CD28− lymphocytes, a population which may contain senescent cells whose impact on immune function may not be benign [31–33]. Such changes are preceded by a measurable age-related decline in the output of αβ+ T cells from the thymus to the naive T cell pool which has been reported in chickens [34], rats [35], mice [36] primates [37] and man [13]. Recent thymic emigrants enter the naive T cell pool where they have a finite lifespan, and this combination of a limited lifespan, reduced thymic output and recruitment into activated and memory T cell pools, contribute to the reduction in the naive T cell pool seen with age.

Current estimations on the timing of cessation of thymic function are imprecise, because they have been derived previously using histological analysis of the thymus combined with phenotypic data on peripheral T cell populations [17,38] and the clear and unambiguous identification of naive T cells in older individuals is difficult [39]. Other means of resolving the issue have been to extrapolate from TREC data derived from studies where the age range was skewed towards younger individuals [14,40,41]. In our study we have looked at sjTREC values in the blood of more than 200 individuals from five different European countries, and our results suggest that between 55 and the mid-80s there appears to be a constant and relatively stable decline in thymic output, which is followed by a significant decline in the 10th decade. Because of the broad distribution area from which the samples were obtained we can discount localized influences, including diet and effects due to pockets of infection causing proliferation in the peripheral T cell pool and subsequent dilution of the sjTREC+ cells. Within the age range analysed there was an apparent gap between the ages 85 and 89; this may be reflective of a poorer quality and smaller proportion of samples collected in this 9th decade. In our study we have seen no significant decline in T cell numbers with age, discounting this as an influential factor, and we have further discounted the effects of gender differences.

Proliferation could contribute towards the differences seen in the 10th decade, although the derivation of the samples from several countries of origin should ameliorate the effects of infection, which may be geographically limited. However, age is also associated with greater proliferation within naive populations [42,43]. While this could also con-
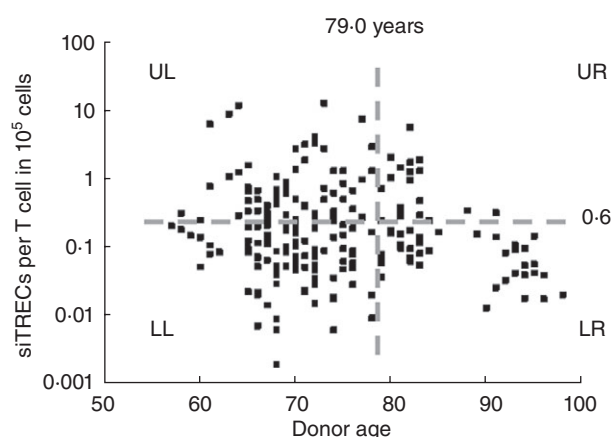

Fig. 4. Signal-joint T cell receptor excision circles (sjTRECs) as a biomarker of immunosenescence. Annotated diagram of the age-related changes observed in sjTREC measurements. The dashed horizontal grey line indicates the median sjTREC per 10^5 T cells in the sample population. The dashed vertical grey line indicates the average life expectancy across the study population (79·0 years). UL, LL, UR, LR refer to the different quadrants formed by the bisection of the data horizontal and vertical lines.

tribute to decline between the 9th and 10th decades it would seem unlikely to account for all of it, as the decline from a value of $2{\cdot}35 \times 10^6$ to $1{\cdot}5 \times 10^5$ would require all the T cells in the body undergoing more than four divisions. The decline could also be due to the loss of the sjTREC from the nucleus due to degradation of the DNA. However, if this occurs we would expect that it should occur at the same rate throughout life. While we cannot resolve whether the decline in thymic output over the entire lifespan is either exponential, biphasic or multiphasic, we have observed a dramatic and precipitous decline in TREC levels starting in the 9th decade. Comparison of the correlation coefficients obtained between the ages of 60–80, 80–90 and those greater than 90 years clearly shows a pronounced change in the rate of decline (Table 2). Despite the apparent discordance with the mean sjTREC levels in Table 1, which indicates an abrupt decline in the 10th decade, both results support the underlying argument that a significant decrease in sjTREC levels is evident by the 10th decade. The possible influences of limited data between the ages of 85–89 years, sample size and mean effects means the precise timing at which the rate declines cannot be calculated. However, it is suggestive that these findings are not attributable to outliers within the sample population. We consider that this may be due mainly to thymic output undergoing a severe decline in the mid-80s to the early 90s years. Such an explanation would also fit with the results from a recent study, which showed that 21 of 25 centenarians had undetectable sjTREC levels [44].

Disclosure

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